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INTRODUCTION

The ongoing goal of this proposal is to determine the possible contribution of the tyrosine kinase, Jak2, in the development and progression of breast cancer. To this end, we have shown that Jak2-null mammary epithelium fails to undergo pregnancy-mediated proliferation or differentiation. Furthermore, we have determined that the upstream prolactin receptor (PrlR) and the down stream transcription factor (Stat5) are also required for functional development of mammary epithelium at pregnancy. Taken together, these data demonstrate that the PrlR-Jak2-Stat5 pathway plays a central role in normal mammary gland development by executing a genetic program leading to the proliferation and differentiation of mammary epithelia cells. Since genetic inactivation of this pathway at multiple levels results in a lack of proliferation, we reasoned that abnormal and constitutive activation of this signaling cascade could lead to the uncontrolled proliferation of mammary cells resulting in the development and progression of tumorigenesis. In this connection, nuclear localization of Stat5a and Stat3 in tumors arising in mice carrying mutations in the *Brca-1* and *p53* genes was apparent. Experiments also revealed that two membrane transporters involved in fluid homeostasis, namely the water transporter aquaporin 5 (AQP5) and the sodium-potassium-chloride cotransporter (NKCC1) are both expressed in mouse mammary tumor tissue derived from mice carrying mutations in the *Brca-1* and *p53* genes. To extend these observations further, analysis of human breast tumor tissue in the form of a multi-sample tissue arrays was performed. In the first set of experiments nuclear Stat5a and Stat3 was apparent in some cells. More recently, a more comprehensive analysis was performed utilizing a human tissue array with associated tumor information. In contrast to mouse mammary tumors, AQP5 was not detected in any human breast tumors. However, normal human breast epithelium showed significant nuclear localization of Stat5a and Stat3. Analysis of over 40 human breast tumors revealed that over 25% of them possessed nuclear Stat3 and/or Stat5 but was independent of tumor type. These data suggest that activation of Stats is a common feature of both normal and tumorigenic human breast tissue.

BODY

Analysis of Jak2/Stat5 protein in normal mouse mammary gland and mammary tumors

The demonstration that transplantation of Jak2-null epithelium failed to undergo pregnancy-mediated proliferation and differentiation provide unequivocal evidence that the Jak2 protein is active in mammary epithelium. Although commercially available antibodies raised against Jak2 are capable of detecting Jak2 in cell lines, Jak2 protein could not be detected in mammary epithelium at any stage of development despite the application of several methods and numerous attempts (Fig. 1A). In contrast, phosphorylated Stat5a protein was readily detected (Fig. 1B). These data suggest that a) the phosphorylation of Jak2 protein is transient and occurs only over a short time period or b) Jak2 protein phosphorylation is unstable and phosphorylation is lost during extraction or c) the level of phosphorylated Jak2 is below the detection of western blotting procedures or d) the antibodies failed to immunoprecipitate Jak2 protein from the prepared extracts.

Figure 1: Analysis of Jak2 and Stat5a phosphorylation status in mouse mammary epithelium at lactation day 10

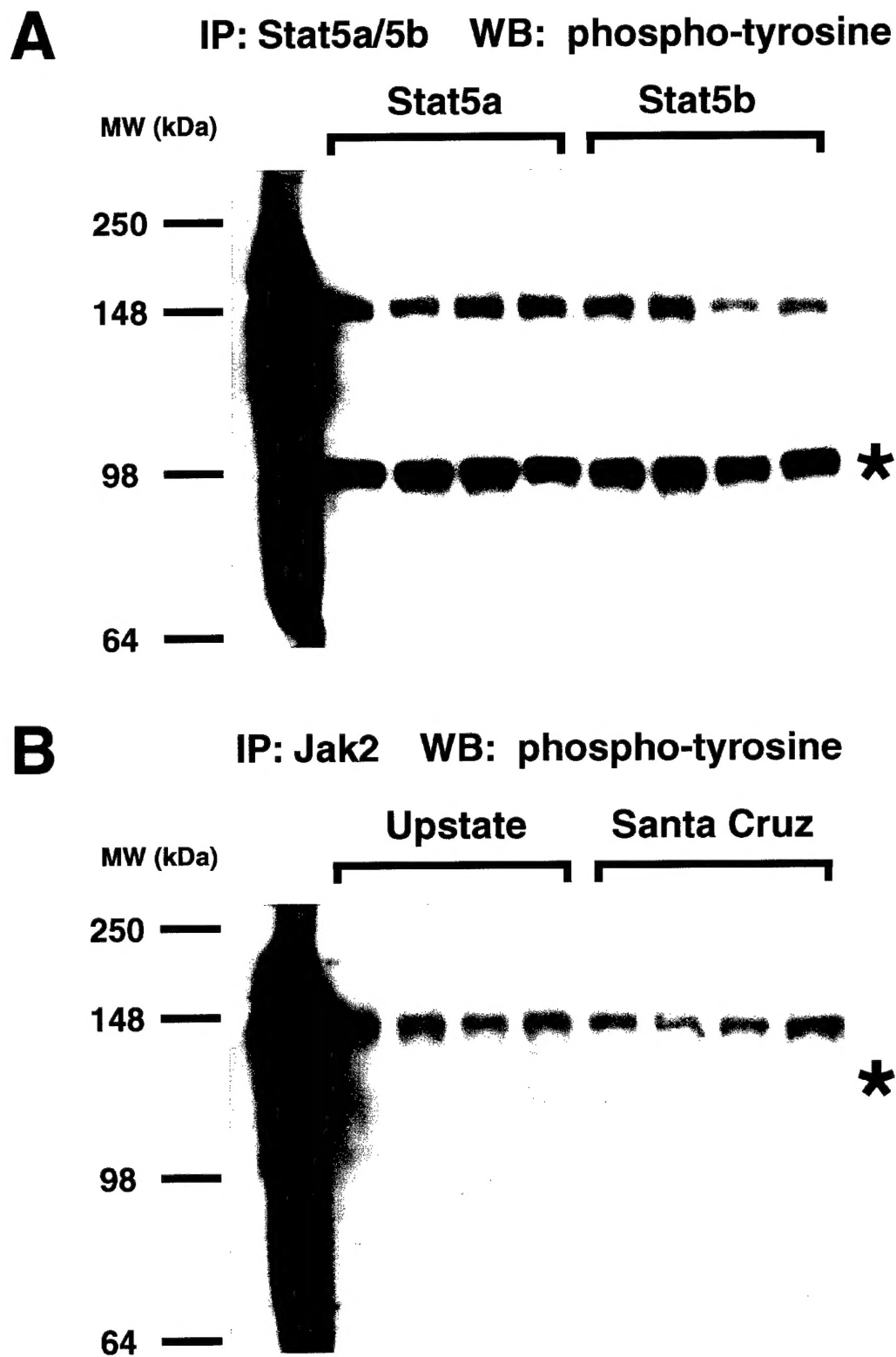


Figure 1: Mouse mammary tissue was removed from female mice at day 10 of lactation and subjected to protein extraction according to standard procedures. Stat5a, Stat5b (A) and Jak2 (B) were immunoprecipitated from the protein extracts using in-house (Stat5a and Stat5b) or commercially available (Jak2; Upstate or Santa Cruz Biotechnology) antibodies. Proteins were electrophoresed on an 8% Tris-Glycine gel and phosphorylated proteins were detected using an anti-phosphotyrosine antibody. * Predicted position of proteins according to molecular weight.

Since mammary epithelium contains numerous cell types, western blotting is not an ideal means to assess specific contribution of the mammary epithelial cells. Therefore, an immunohistochemical approach was employed to assess the localization of Stat5a and Stat3 proteins. It is well established that the phosphorylation of Stat monomers results in their dimerization and subsequent translocation to the nuclear compartment (reviewed in [1]). Therefore, the presence of nuclear Stat protein is an indication of their functional status. Ordinarily, Stat5a phosphorylation is first observed in mouse mammary gland at mid pregnancy and persists throughout lactation (ref. and Fig. 1B) but is rapidly lost at involution [2]. In contrast, Stat3 phosphorylation is observed within 24 h of pups being removed from the mothers. This phosphorylation event coincides with the translocation of Stat3 to the nucleus (see [3]) and is associated with programmed cell death or apoptosis. Through the analysis of mouse models that over express Stat5 [4], lack functional Stat5 [5, 6] and an oncogenic mouse model crossed into a Stat5-null background [7] it is clear that Stat5 is involved in proliferation and can contribute to tumorigenesis. Furthermore, constitutive activation of Stat3 has been associated with tumorigenesis (reviewed in [8]). In this connection it was previously shown that both nuclear Stat3 and Stat5a could be detected in mammary tumors arising in *Brcal/p53* mutant mice independent of lactation status suggesting that Stat5a and Stat3 phosphorylation may contribute to tumorigenesis in this mouse model. Since Jak2 can phosphorylate both Stat5a and Stat3 these data imply that Jak2 may play a central role in tumor progression.

To extend these observations further, the specific localization of Stat3 and Stat5a in other oncogenic mouse models was assessed (Fig. 2 – 3). MMTV-Neu mice [9] develop mammary tumors within 5 – 7 months. Furthermore, recent expression profiling data have revealed that neu-induced mammary tumors exhibit genetic and morphological similarities to ErbB2-expressing human breast cancers [10]. Interestingly, crossing MMTV-Neu mice into a Stat5a-null background resulted in a significant delay in tumorigenesis, providing evidence that Stat5a contributes to tumor progression in this mouse model (unpublished data). To examine whether other Stat family members might play a role in tumor formation in the absence of Stat5a the immunolocalization of Stat3 was determined. As expected, Stat5a protein was not detected (Fig. 2B, D, F). However, significant nuclear and cytoplasmic accumulation of Stat3 was observed (Fig. 2A, C, E) suggesting that the activation of Stat3 may compensate for the absence of Stat5a and thus is a potential contributing factor in tumor progression. Female MMTV-Wnt1 mice develop mammary and salivary gland adenocarcinomas within 5 – 7 months [11]. We have also observed mammary tumor formation within 6 months in mice that express hepatocyte growth factor (HGF) under the control of the whey acidic protein (WAP) promoter (in press). To evaluate the potential role of the Jak-Stat pathway in these oncogenic mouse models, the localization of Stat3 and Stat5a was determined (Fig. 3). In two separate tumors isolated from MMTV-Wnt1, both nuclear Stat3 and Stat5a were observed (Fig. 3, A-D, white arrowheads). Similar nuclear localization of Stat3 and Stat5a was apparent in mammary tumor tissue dissected from WAP-HGF-expressing mice (Fig. 3, E-H).

Taken together, it is demonstrated that the nuclear localization of Stat3 and Stat5a is apparent in three separate oncogenic mouse models (MMTV-neu, MMTV-Wnt1 and WAP-HGF) suggesting that activation of genetic pathways induced by these transcription contribute to tumor development and, most likely, tumor progression.

Figure 2: Localization of Stat3 and Stat5a in mouse mammary tumors induced by an MMTV-neu transgene in a Stat5a-null background.

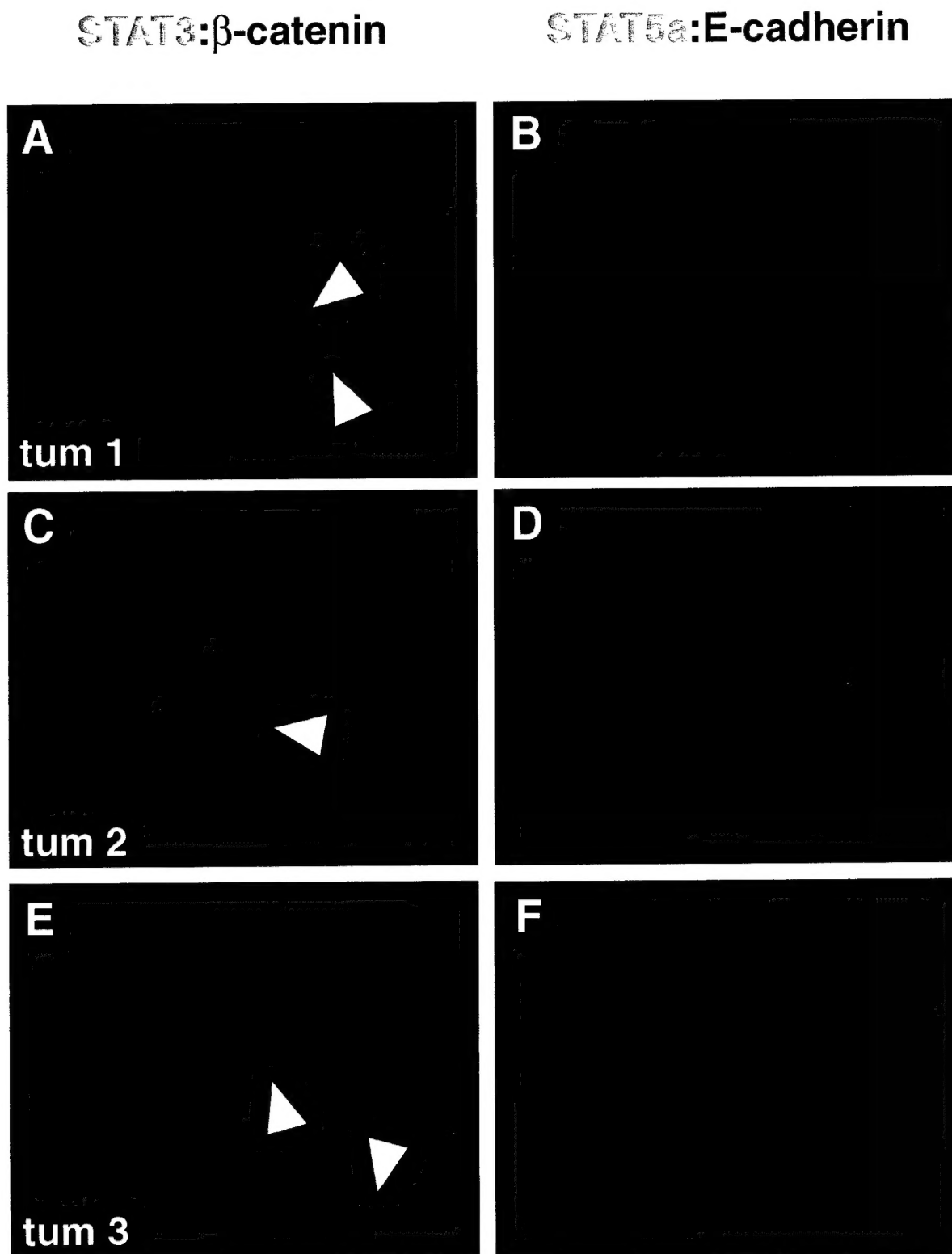


Figure 2: Palpable mammary tumors were excised from mice expressing the *Neu* gene under the control of the MMTV promoter in a Stat5a-null background. Tumor tissue was fixed, embedded in paraffin and sectioned. Sections were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against Stat3 (green) and β -catenin (red) (A, C, E) or Stat5a (green) and E-cadherin (red) (B, D, F). Fluorescent-conjugated antibodies were applied and representative images were captured. Stat3 was observed both in the nucleus (white arrowheads) and the cytoplasm (yellow arrowheads) of tumor cells. Note the absence of Stat5a staining, indicative of the Stat5a-null background (B, D, F).

Figure 3: Localization of Stat3 and Stat5a in mouse mammary tumors induced by WAP-HGF and MMTV-Wnt1 transgenes.

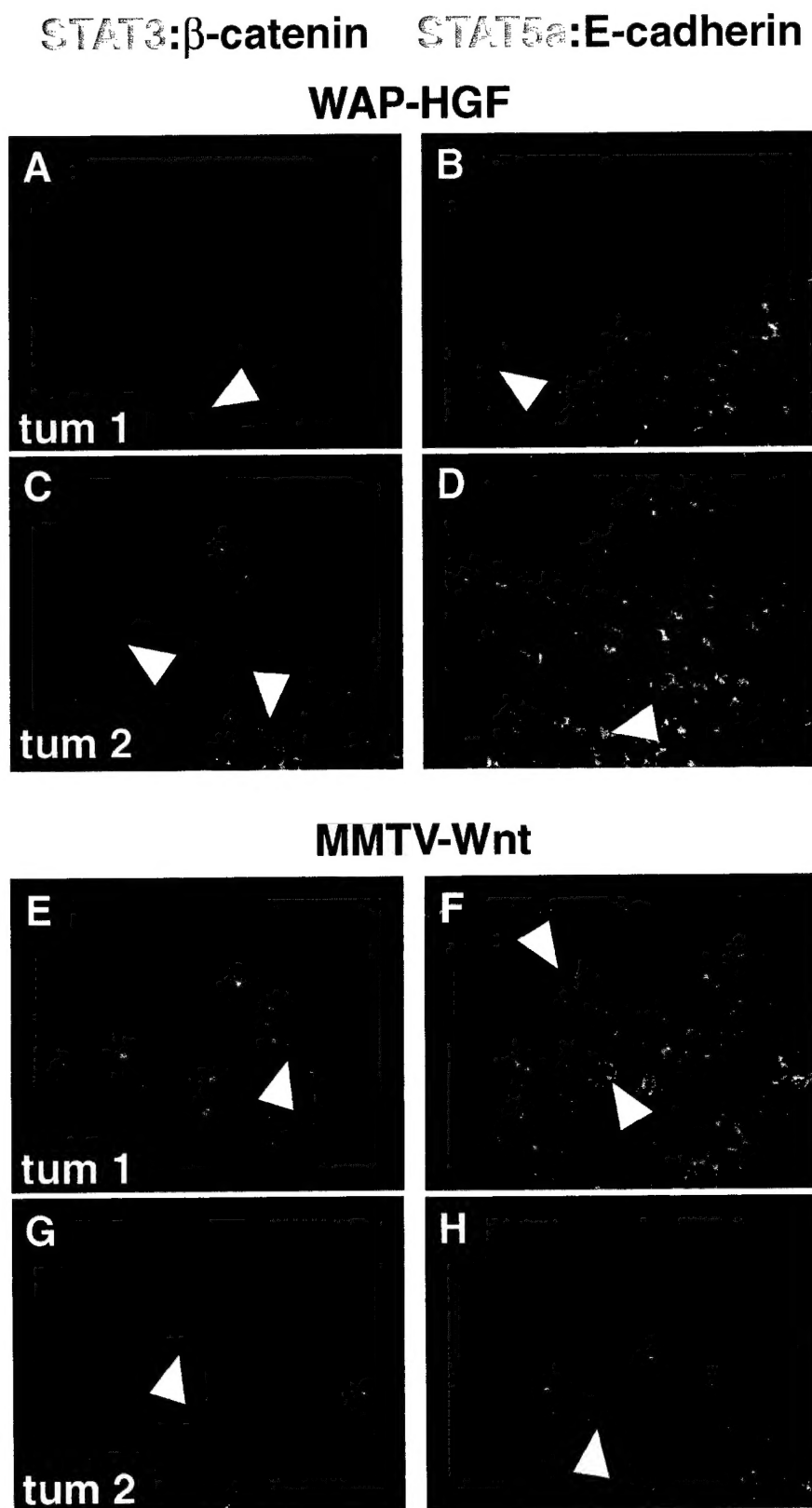


Figure 3: Palpable mammary tumors were excised from mice expressing the *HGF* (A – D) or *Wnt* (E – H) genes under the control of the WAP and MMTV promoters, respectively. Tumor tissue was fixed, embedded in paraffin and sectioned. Sections were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against Stat3 (green) and β -catenin (red) (A, C, E) or Stat5a (green) and E-cadherin (red) (B, D, F). Fluorescent-conjugated antibodies were applied and representative images were captured. Stat3 and Stat5a were observed both in the nucleus (white arrowheads) and the cytoplasm (yellow arrowheads) of tumor cells.

Analysis of Stat3 and Stat5a protein in normal human breast and human breast cancer samples

Although mammary tumors arising in both the *Brca1/p53* mutant mouse model and *neu*-induced mouse models are similar in pathology to those observed in their respective human breast cancer counterparts, the translation of data obtained in the mouse is no substitute for data obtained from actual human breast cancer samples. To this end, TARP tissue arrays (http://ccr.cancer.gov/tech_initiatives/tarp/) were utilized to examine the localization of Stat3 and Stat5a in human breast cancer tissue samples. Although nuclear Stat3 and Stat5a were observed in some human breast tumor samples in the initial tissue arrays examined overall interpretation was limited due to the absence of normal human breast epithelium for comparative purposes. Over the past year several commercially available tissue arrays have become, including human breast cancer-specific tissue arrays.

To further examine the potential role of Stat3 and Stat5 in tumor development and extend the previous analysis the localization of Stat5a (Fig. 4) and Stat3 (Fig. 5) was determined. Of note is the presence of significant nuclear Stat5a in tissue sampled from normal mouse mammary gland at lactation (Fig 4A, white arrowhead) and normal human breast (Fig. 4 A and B, white arrowheads). Nuclear Stat5a was also observed in some human tumor samples (Fig. 4 F, I, J and L, white arrowheads) but completely absent from others (Fig. 4 D, G and H). Since the presence of Stat5a is a hallmark of mammary epithelium it is likely that its absence in some tumors corresponds with the differentiation status of the tumor sample. Analysis of Stat3 localization gave similar results (Fig. 5). Thus localization of Stat3 in the nucleus was observed in normal mouse mammary gland at involution (Fig. 5A, white arrowhead) and in normal human breast (Fig. 5 A and B, white arrowheads). Similar to the Stat5a staining, nuclear Stat3 was observed in some tumors (Fig. 5, D, E, F, I and J, white arrowheads) but not others (Fig. 5, G and L). Interestingly, some tumors expressed both nuclear Stat3 and nuclear Stat5a (Fig. 4 and 5, I, J and L) suggesting that the constitutive activation of multiple Stat family members may contribute to tumor development and progression.

Figure 4: Localization of Stat5a in human breast cancer samples.

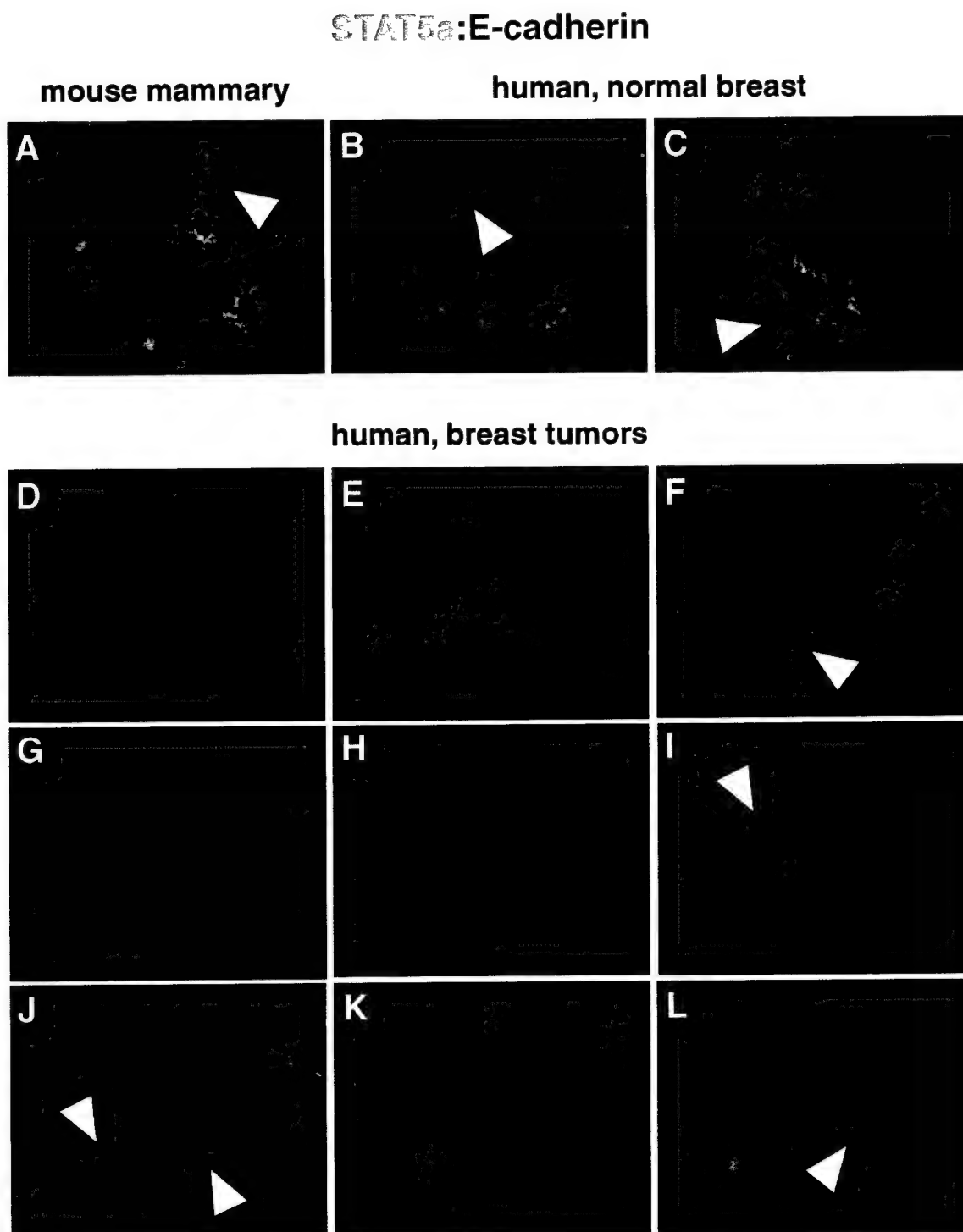


Figure 4: Human breast cancer tissue arrays were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against Stat5a (green) and E-cadherin (red). Fluorescent-conjugated antibodies were applied and representative images were captured. Stat5a was observed both in the nucleus (white arrowheads) and the cytoplasm (yellow arrowheads) in some tumor cells but not others.

Figure 5: Localization of Stat3 in human breast cancer samples.

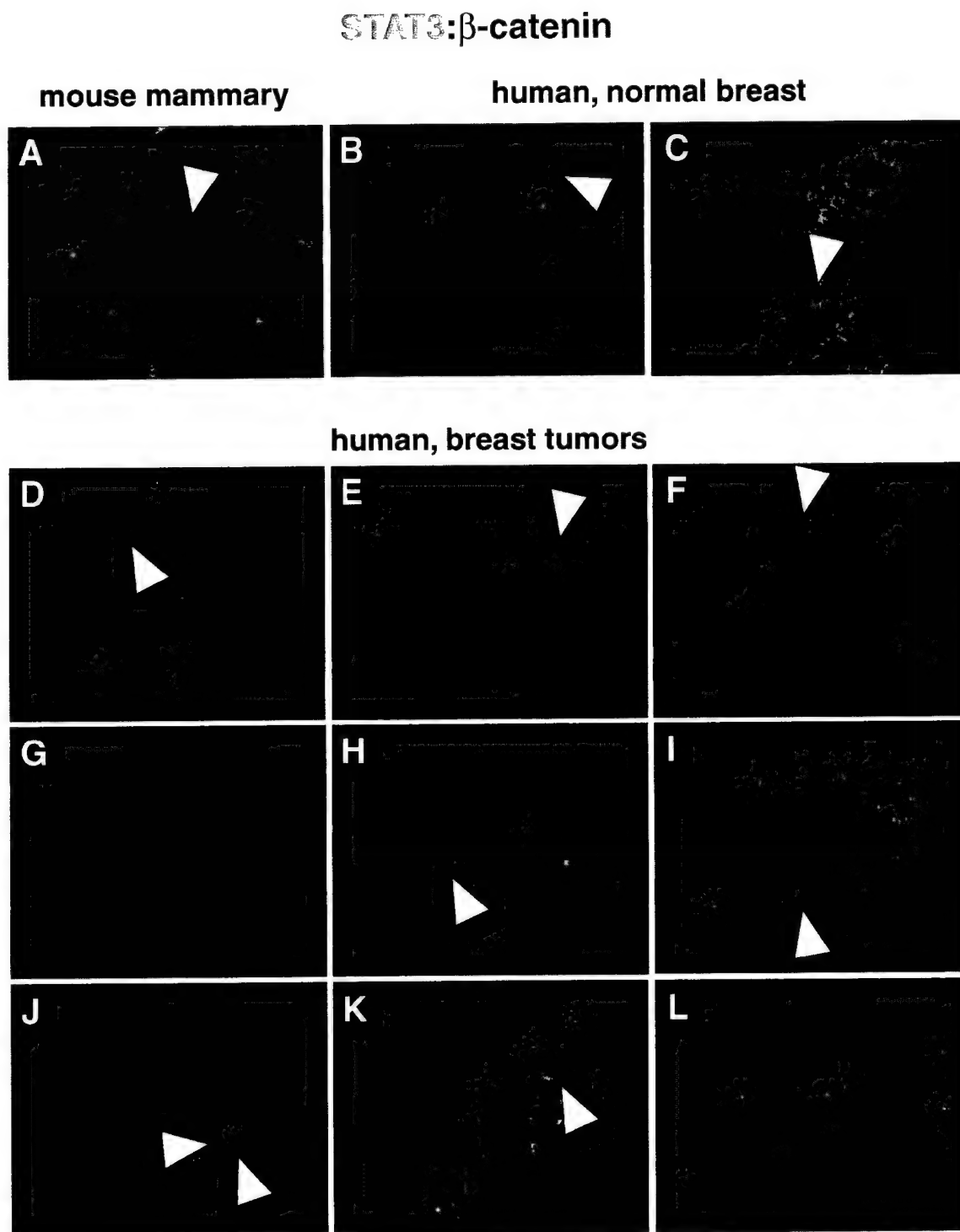


Figure 5: Human breast cancer tissue arrays were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against Stat3 (green) and β -catenin (red). Fluorescent-conjugated antibodies were applied and representative images were captured. Stat5a was observed both in the nucleus (white arrowheads) and the cytoplasm (yellow arrowheads) in some tumor cells but not others.

Generation of a mouse model carrying a conditional deletion of the Jak2 gene

The inactivation of the Jak2 gene results in embryonic lethality due to a lack of definitive erythropoiesis [12], which does not permit the assessment of Jak2 function in mammary epithelium by conventional means. Therefore, one of the goals of the proposal was to generate a mouse model in which the Jak2 gene could be inactivated specifically in the mammary epithelium, thereby circumventing the deleterious effect of systemic Jak2 gene deletion.

It was reported previously that problems were initially encountered with the Jak2 genomic targeting construct, which failed to produce many ES colonies subsequent to injection. Following reengineering of the construct injection of the construct resulted in over 250 colonies. These colonies were pre-screened by PCR to select clones containing the 5' loxP site. The positive clones that were identified in this screen were then selected for further analysis by Southern blot. Of the 43 positive clones screened, 5 were identified as possible candidates that had undergone homologous recombination. Unfortunately, none of the 5 clones selected resulted in a conditional allele.

Generation of *Brcal/p53* mutant mouse model in a Jak2-null background

Since the successful generation of a Jak2 conditional mouse model was not achieved, the subsequent generation of a *Brcal/p53* mouse model in a Jak2-null background was not possible. As a word of caution for future studies proposing to use the *Brcal/p53* mutant mouse model with the intention of crossing it into another mouse model it should be noted that the *Brcal* and *p53* genes are closely linked on chromosome 11. Therefore, a huge number of crosses would have to be preformed in order to generate a mouse carrying a conditional *Brcal* allele and the Cre-recombinase in the *p53* +/- background. Taking this potential problem into account and also the availability of numerous other oncogenic mouse models it might serve better to cross the Jak2-null mice into these models. In particular, since it has been demonstrated that MMTV-Neu, MMTV-Wnt1 and WAP-HGF tumors present with significant nuclear Stat5a and Stat3 these models might be more appropriate.

AQP5 expression in mouse mammary tumor tissue

Screening of the mouse EST database with full-length clones representing NKCC1 and AQP5 revealed high expression in EST libraries derived from mouse mammary tumors and 18 of the top 50 clones (36%) and 38 of the top 50 clones (76%) were of tumor origin, respectively. Furthermore, analysis of NKCC1 and AQP5 protein demonstrated their presence in tumors derived from *Brcal/p53* mutant mice suggesting they may be candidates for novel tumor-associated proteins. Given the role of both these membrane transporters in fluid transport it is tempting to speculate that they might serve a protective effect for tumors in the context of preventing tumor oedema and therefore promote tumor survival (see [13, 14]).

To extend these observations further on a genetic level the expression of AQP5 mRNA was determined in a variety of tumors isolated from oncogenic mouse models (MMTV-Neu, MMTV-Wnt1 and MMTV-PyV mT mice, see [15]). As can be seen in

Fig. 6A, expression of AQP5 mRNA was observed in tumors derived from all three models. Furthermore, the presence of AQP5 mRNA was independent of tumor developmental stage as determined by analysis of staged tumor samples (Fig. 6B). These data provide further evidence that AQP5 is a potential tumor-associated gene. However, the analysis of AQP5 protein in human breast tumor samples via immunohistochemistry failed to reveal any significant AQP5 expression in the samples analyzed (data not shown).

The use of protein markers in the analysis of mammary gland development

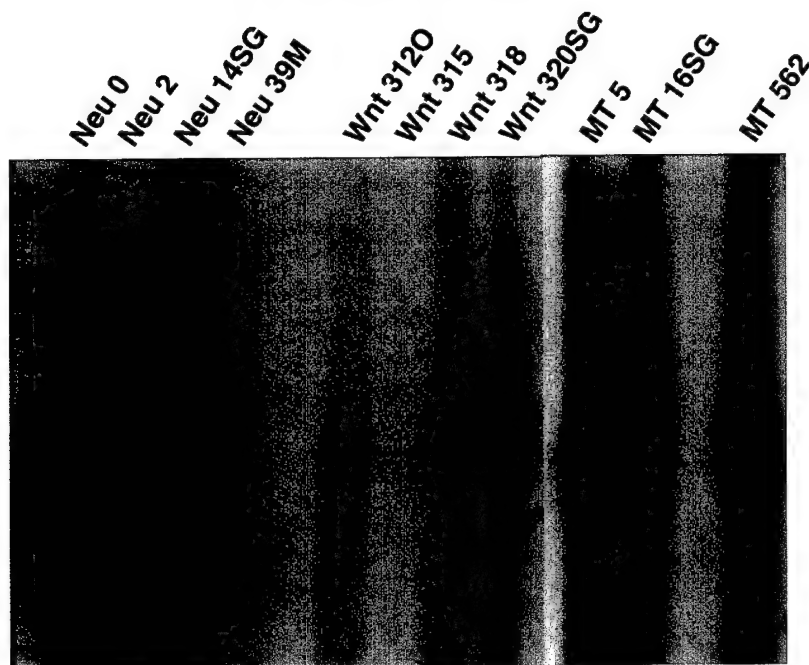
Using immunohistochemical methods, it was demonstrated that the lack of proliferation and differentiation in Jak2-null and Stat5-null mammary transplants parallels the maintenance of high levels of two ductal markers (NKCC1 and AQP5) and the absence of a secretory marker (Npt2b). These data suggested the intriguing possibility that these markers might be applicable to the analysis of mammary gland defects in other mouse models. In order to evaluate this possibility, several different mouse models that present with deficiencies in mammary gland development were analyzed (see published paper 1). The replacement of serine residues in the activation loop of IKK α results in a failure of females to lactate and nurse their young [16]. Analysis of the secretory marker Npt2b established that lactating glands lacked detectable Npt2b (Fig. 7B) as compared to wild type glands (Fig. 7A). Interestingly, this phenotype was linked to a reduction in cyclin D1 mRNA levels, as determined by northern blot analysis. Therefore the IKK α mutant animals were crossed with animals over expressing cyclin D1, which resulted in restoration of a functional lactating gland as assessed by histological analyses and pup mortality [16]. In addition to the functional rescue observed, immunohistochemical experiments revealed a complete restoration of apical Npt2b protein (Fig. 7D). These data demonstrate that the induction of apical Npt2b protein is associated with the attainment of full secretory function. Another mouse model in which inhibin β B is inactivated is also unable to successfully lactate [17]. However, the determination of Npt2b protein revealed normal levels (Fig. 7E). Since normal Npt2b protein levels were observed it is unlikely that the functional status of the inhibin β B-null epithelium is affected but rather the phenotype can be explained due to much reduced mammary epithelial cell proliferation and expansion. In another mouse model in which the ErbB4 gene was knocked out specifically in the mammary gland (see published paper 2) female dams were unable to successfully lactate (Fig. 8). Although downregulation of NKCC1 levels was observed at pregnancy and lactation in both wild type and ErbB4 KO glands (Fig. 8A – F), the establishment of apical Npt2b was not observed in ErbB4 KO glands at lactation (Fig. 8L) as compared to wild type (Fig. 8K).

Figure 6: Detection of AQP5 mRNA in different mouse mammary tumor samples.

Northern blot: AQP5 probe

A

tumor models



B

tumor stages

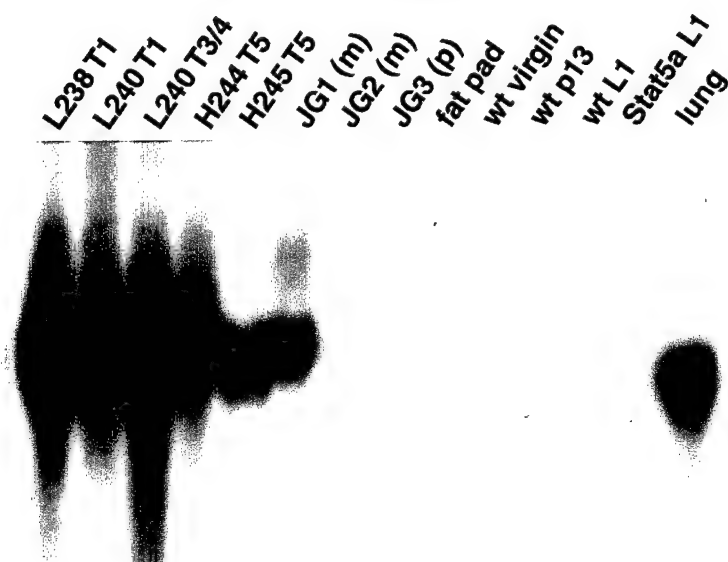


Figure 6: Total RNA was isolated from palpable tumors derived from (A) mice expressing a variety of oncogenes and (B) different stages of tumor development and probed for the expression of AQP5. (A) AQP5 RNA was detected in tumors derived from MMTV-Neu (Neu), MMTV-Wnt1 (Wnt) and MMTV-PyV mT (MT) tumors (SG, salivary gland; M, male; O, ovary). (B) AQP5 RNA was detected in all tumors staged according to developmental progression based on histological comparisons (T1 – T2, low grade; T3, middle grade; T4 – T5, high grade).

Others [18] and we [19] have demonstrated that deletion of the Stat3 gene results in delayed mammary gland involution. In an attempt to assess the functional consequence of Stat3 deletion the localization of Npt2b protein was determined (Fig. 8). Although no difference was observed at day 10 of lactation (cf. Fig. 8A and 8D), a significant difference was observed at day 2 (cf. Fig. 8B and E) and day 6 (cf. Fig. 8C and F) of involution in wild type vs. conditional Stat3 fl/fl WC samples. Therefore the observed delay in involution, as assessed by histological analyses, was supported by the persistence of significant apical Npt2b protein in conditional Stat3 fl/fl WC glands. To further establish the lactational competency of the Stat3 fl/fl WC epithelium, pups were removed from lactating dams for 6 days. Subsequently four 5-day-old pups were put back on the mothers to see if they could reestablish lactation. Interestingly, of 20 pups only 1 perished when placed back onto 6-day-involuting Stat3 fl/fl WC mothers. In contrast, none of the pups placed back onto 6-day-involuting wild type mice survived [19]. To determine whether functional lactation was reestablished in Stat3 fl/fl WC the analysis of Npt2b was ascertained. Fig. 8J demonstrates that, as compared to resuckled wild type epithelium (Fig. 8I), the functional rescue of Stat3 fl/fl WC epithelium corresponded with the apical expression of Npt2b protein.

Figure 7: Analysis of Npt2b and E-cadherin expression in IKK α knockin mice and inhibin β B-null mice.

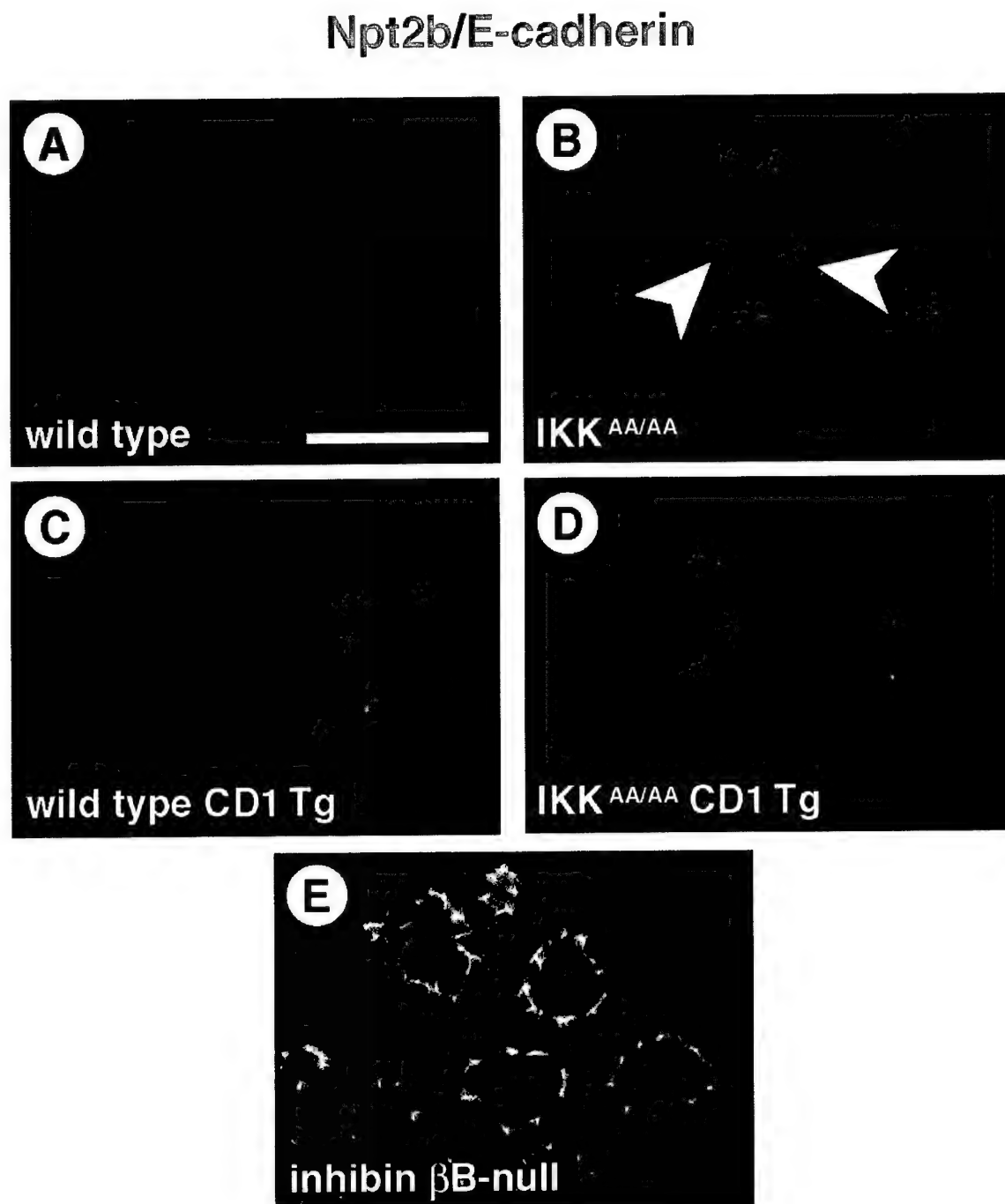


Figure 7: Mammary tissue samples were isolated from (A) wild type, (B) IKK α knockin, (C) cyclin D1 transgenic and (D) IKK α knockin/cyclin D1 transgenic mice at lactation day 1. Samples were fixed, embedded in paraffin and sectioned. Sections were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against E-cadherin (green) and Npt2b (red). Fluorescent-conjugated antibodies were applied and representative images were captured. Note the absence of apical Npt2b protein in IKK α knockin samples (B, white arrowhead) and restoration of Npt2b in IKK α knockin/cyclin D1 transgenic samples (D).

Figure 8: Analysis of NKCC1/SMA and Npt2b/E-cadherin in wild type and ErbB4-null mice.

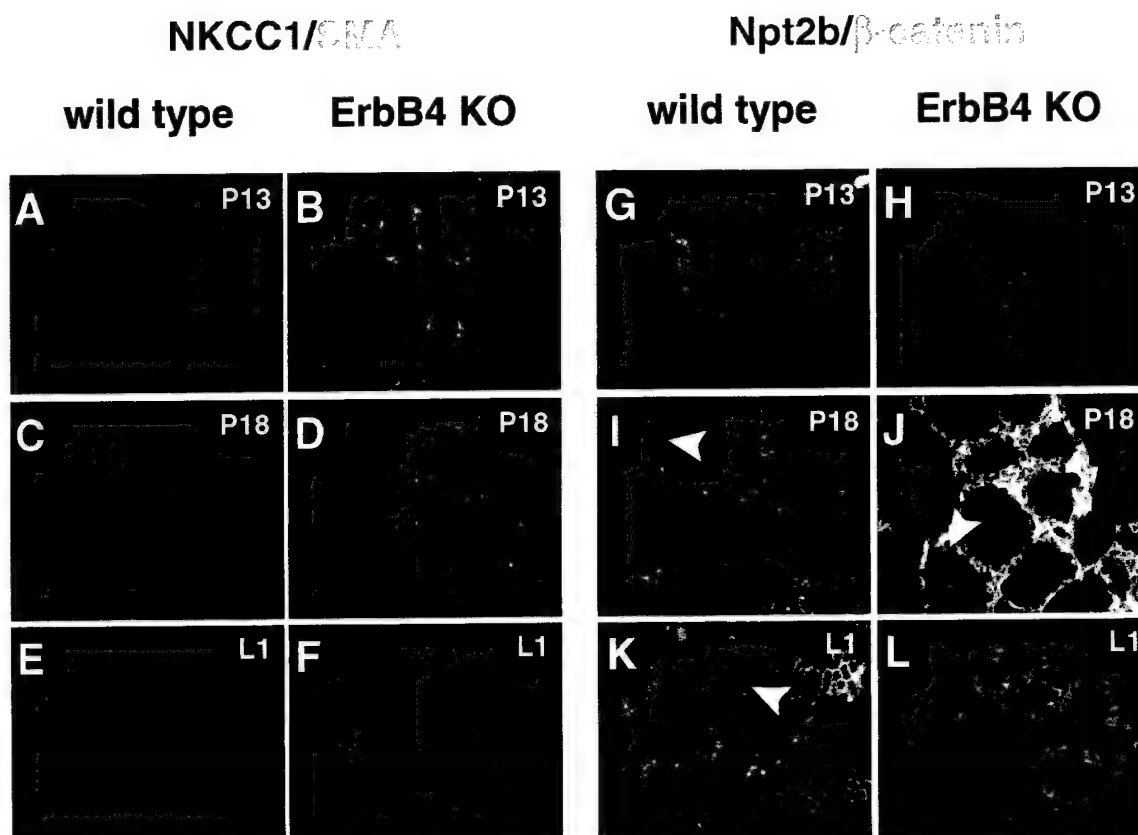


Figure 8: Mammary tissue samples were isolated from wild type (A, C, E, G, I and K), and ErbB4 KO (B, D, F, H, J and L) mice at pregnancy day 13 (P13), pregnancy day 18 (P18) and lactation day 1 (L1) as indicated. Samples were fixed, embedded in paraffin and sectioned. Sections were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against NKCC1 (red) and smooth muscle actin (green) (A-F) or Npt2b (red) and E-cadherin (green) (G-L). Fluorescent-conjugated antibodies were applied and representative images were captured. Note the absence of apical Npt2b protein in the ErbB4 KO sample at lactation day 1 (L) as compared to wild type (K, white arrowhead).

Figure 9: Analysis of Npt2b and E-cadherin expression in conditional Stat3-null mice.

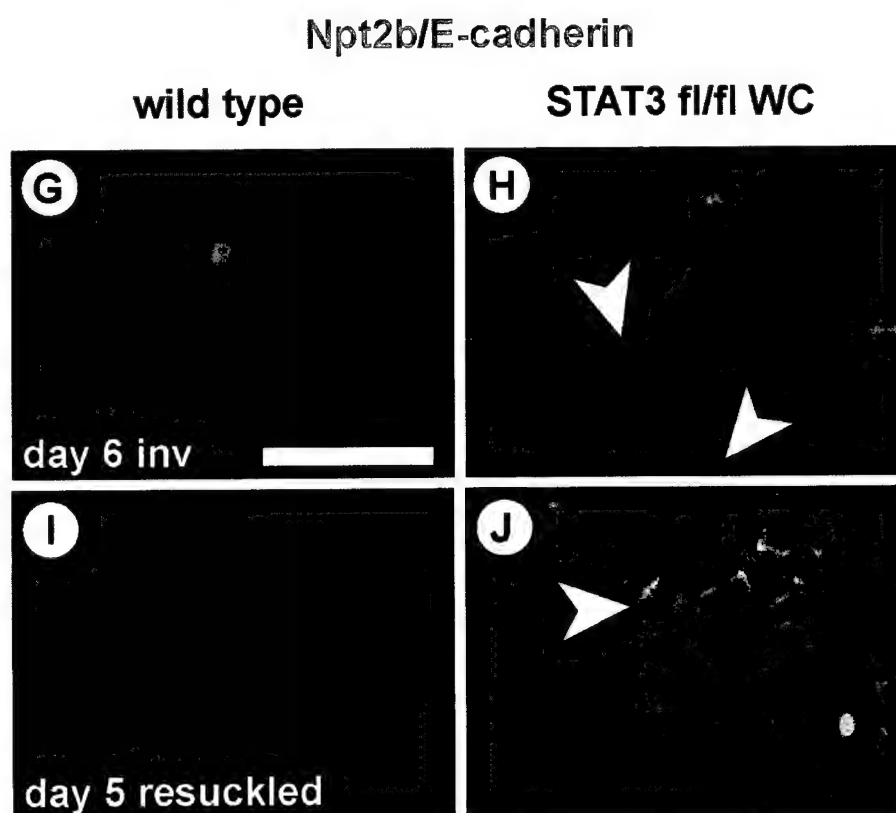
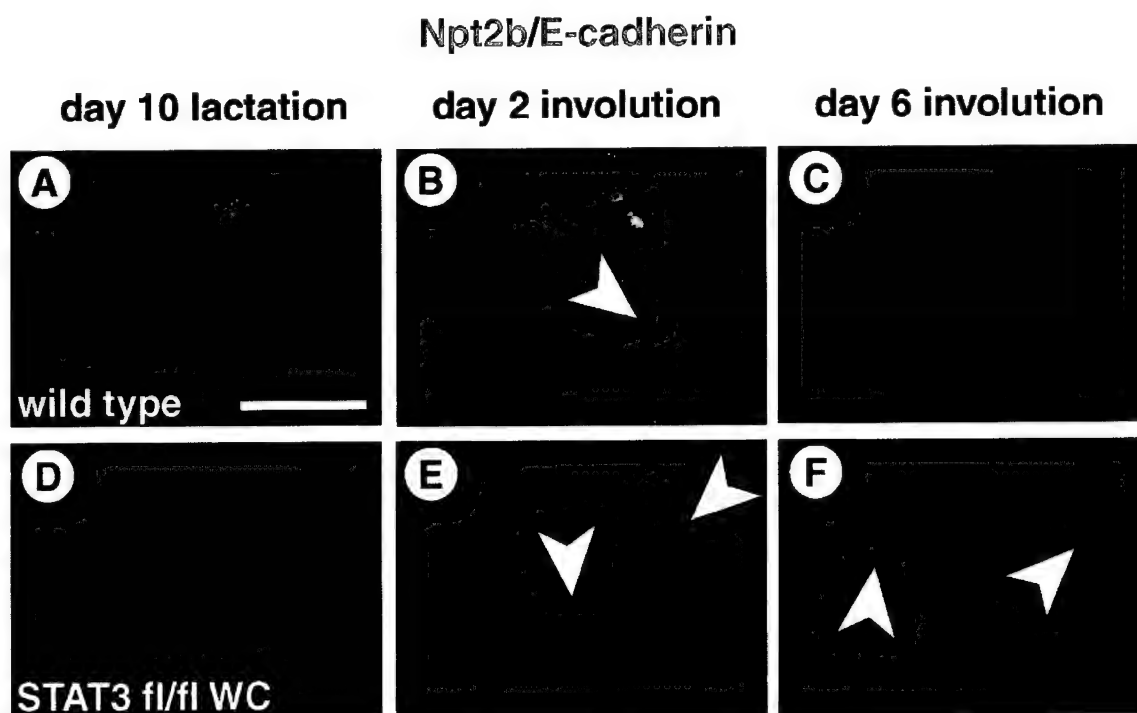


Figure 9: Mammary tissue samples were isolated from (A – C, G, I) wild type and (D – F, H, J) Stat3 fl/fl WC mice. Samples were fixed, embedded in paraffin and sectioned. Sections were cleared in xylene, rehydrated and subjected to antigen retrieval procedures. Subsequent to blocking, sections were incubated with antibodies raised against E-cadherin (green) and Npt2b (red). Fluorescent-conjugated antibodies were applied and representative images were captured. Note the persistence of apical Npt2b protein in Stat3 fl/fl WC samples at day 2 of involution (E, white arrowhead) versus wild type (B). Apical Npt2b protein is evident in the Stat3 fl/fl WC sample upon 5 days resuckling of 6-day-involuting tissue (J, white arrowhead) but not wild type sample (I).

KEY RESEARCH ACCOMPLISHMENTS

- Further evidence of nuclear Stat5a and Stat3 in the oncogenic mouse models MMTV-Neu, MMTV-Wnt1 and WAP-HGF.
- Further evidence of nuclear Stat5a and Stat3 in human breast cancer tissue samples.
- Evidence of AQP5 mRNA expression in tumors derived from MMTV-Neu, MMTV-Wnt1 and MMTV-PyV mT mice and different stages of tumor development.
- The application of protein markers (proteotyping) to the analysis of mammary defects in numerous mouse models.

REPORTABLE OUTCOMES

Published papers:

1. **Shillingford, J.M.**, Miyoshi, K., Robinson, G.W., Bieri, B., Cao, Y., Karin, M. and Hennighausen, L (2003). Proteotyping of mammary tissue from transgenic and gene knockout mice with immunohistochemical markers: a tool to define developmental lesions. *J Histochem Cytochem* **51**:555-565.

2. Long, W., Wagner, K-U., Lloyd, K.C., Binart, N., **Shillingford, J.M.**, Hennighausen, L. and Jones F.E. (2003). Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of Stat5. *Development* **130**:5257-5268.

CONCLUSIONS

Analysis of oncogenic mouse models and human breast cancer tissue samples has revealed the nuclear localization of Stat3 and Stat5a, further suggesting that genetic pathways induced by these transcription factors contribute to tumor development and progression. In addition, the localization of AQP5 protein and presence of AQP5 mRNA in a variety of oncogenic mouse models suggest that AQP5 (and NKCC1) expression may somehow be linked to tumor development. It is tempting to speculate that tumors recruit these membrane transporters to benefit their growth and survival by regulating tumor oedema. Although the generation of a Jak2 conditional knockout mouse model was unsuccessful, the large body of data in the literature and the work presented in this report strongly suggest that signaling via the Jak-Stat pathway and its ability to impinge on cellular proliferation and apoptosis plays a fundamental role in the tumorigenic process. Finally, through the use of defined protein markers, it is demonstrated that the cell localization and expression pattern of NKCC1, AQP5 and Npt2b can be used to assess specific developmental defects in mammary gland development in numerous mouse models.

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